THE EFFECT OF THE PROTEASE INHIBITOR RITONAVIR ON THE RATE OF METABOLISM OF MIDAZOLAM

George Haritos

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ABSTRACT

Midazolam (MDZ) is a benzodiazepine administered for preoperative sedation. ***Protease inhibitors*** (PI) such as ***ritonavir*** (RIT) are drugs used in the treatment of the human immunodeficiency virus (***HIV***) and acquired immune deficiency syndrome (***AIDS***). They act as inhibitors of HIV-1 protease, a major enzyme responsible for production and maturation of infectious viral progeny. A recent case study discussed prolonged hypnotic effects of MDZ when administered to a patient on a PI regimen. MDZ and RIT biotransformation is accomplished by the P450 3A4 enzyme, suggesting a drug interaction may occur. The purpose of this study was to assess the in vitro metabolic reactions of MDZ in the presence of RIT. Human liver microsomes (HLM) from four cadaver samples were prepared and pooled to provide a homogenous mixture of P450 isozymes. They were incubated with MDZ alone and then in combination with RIT. Therapeutic concentrations of MDZ were used (0.5, 1, 3, 6, 12μM). Subtherapeutic RIT concentrations of 0.005, 0.01, 0.05, 0.1 μM were used, as pilot studies demonstrated complete inhibition above 0.1μM. Through the use of high performance liquid chromotography (HPLC), the ratio between MDZ's major metabolite and the internal standard were obtained. Significant inhibition of MDZ ***metabolism*** was demonstrated at even subtherapeutic concentrations of RIT. Kinetic analysis showed RIT with an apparent Ki (constant of inhibition) value of 0.00956μM. Percent inhibition was as high as 95%. These values suggest the possibility of prolonged sedation and respiratory depression in the clinical setting.

Key Words: **midazolam protease inhibitor ritonavir HIV AIDS metabolism**

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George Haritos, CAPT, USAF

THESIS

Presented to the Graduate School of Nursing Faculty

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Health Sciences in Partial Fulfillment

of the Requirements for

the Degree of

MASTER OF SCIENCE

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES

October 1999

FOREWORD

The inspiration for this study comes from Dr. E. J. McCarthy. She presented to both Capt B. Todd and myself the importance of this research to our profession. Capt K. Hinkle pioneered these efforts by forging a relationship with the Department of Clinical Pharmacology at USUHS and assisting with the initial direction that this study would take. The cooperation and professional interaction that resulted between the Graduate School of Nursing and the Department of Clinical Pharmacology fostered the collegial atmosphere necessary to conduct this research. Dr. L. Cantilena was instrumental in not only providing resources but assisting in the design of the study and for that I am truly grateful. Svetlana Chertsniakova's remarkable ability in the lab and genuine desire to conduct research was absolutely essential in accomplishing this study. The cooperation exhibited at USUHS is thriving and research continues with the class of 2000.

DEDICATION

God's greatest blessing to me is my family and to them I dedicate this work. They are my inspiration, motivation and encouragement.

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I would like to acknowledge certain individuals that were instrumental in this endeavor. Dr. E. J. McCarthy for the support and direction she gave us in this area of research. Dr L. Cantilena for providing complete access to his facilities and lending us the guidance necessary to complete this study. Dr. E. Levine for his encouragement and review. Sveta Cherstniakova's remarkable abilities in the fields of pharmacology and biochemistry. My colleagues Capt B. Todd and LCDR M. Sanchez; we spent many hours away from our families to complete our studies.

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CHAPTER I. INTRODUCTION

Background of the Problem

Midazolam has been used in the United States since 1986 and is frequently administered in the practice of nurse anesthesia. It is considered safe and effective, however, there have been reports that its effects can be enhanced and negative outcomes have been reported in the presence of other drugs (Merry, Mulcahy, Barry, Gibbons & Back, 1997). Although it has been known that metabolism of midazolam varies under certain conditions, the rate of metabolism has not been investigated in the presence of protease inhibitor drugs until now. Protease inhibitor drugs are presently the most effective treatment for Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS). This in vitro study demostrates that the metabolism of midazolam is extremely inhibited in the presence of ritonavir even at sub therapeutic concentrations.

Properties of Midazolam

Anesthetists strive to provide their patients safe and effective relief of discomfort throughout many medical procedures. The Council on Certification of Nurse Anesthetists (CCNA) (1996) found that midazolam is the second most frequently administered anesthetic agent in the United States today. This drug acts upon the central nervous system as a depressant and is prescribed for preoperative sedation and amnesia of preoperative events (Omoigui, 1995). It is also used for conscious sedation during short diagnostic and endoscopic procedures and in the critical care setting (Fragen, 1997). A unique and important property of midazolam is that it provides patients with antegrade

amnesia allowing them to forget painful and unpleasant procedures. This drug provides a rapid onset of sedation and amnesia with a relatively short half-life of 1.5 to 3 hours (Allonen, Ziegler & Klotz, 1981; Dundee, Haliday, Harper & Brogden, 1984).

Midazolam can pose harmful effects if not administered carefully. The Food and Drug Administration (FDA) issued warnings regarding its use with other drugs in 1987 due to 17 reports of respiratory, apnea, and other problems in patients who had received midazolam and other central nervous system depressants (FDA, 1987). The FDA stressed that patients receive careful monitoring after the administration of midazolam (FDA, 1988).

Studies have described antibiotics such as erythromycin and other calcium agonist drugs as inhibitors of the metabolism of midazolam. The studies have shown that midazolam and these other drugs use the P450 3A system for their metabolism and thus may increase bioavailability of midazolam (Fabre et al., 1988, Kronbach, Mathys, Umeno, Gonzales & Mayer, 1989; Olkkola et al., 1993). Thus, there is a decrease in plasma clearance of midazolam, which prolongs and increases the hypnotic and respiratory depressant effects.

Protease Inhibitors

Protease inhibitor drugs are being prescribed for the treatment of individuals infected with Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiencey Syndrome (AIDS). The spread of HIV/AIDS continues to infect many people in the world today (Centers for Disease Control [CDC], 1997). As a result, there is a rise in the use of protease inhibitors. Protease inhibitors interrupt the pathway HIV uses within a

cell to create more virus (Deeks, Smith, Holodniy & Kahn, 1997). Once HIV is introduced into a cell, it attempts to replicate and invade other cells. The virus accomplishes this by replicating itself via the proteins reverse transcriptase and protease. A protease inhibitor attempts to halt the protease from assisting in the assembly of a new virus. Protease inhibitors are often combined with other antiretroviral drugs such as zidovudin (AZT) and didanosine (ddI) to diminish the antiviral load. Protease inhibitors utilize the P450 3A system for metabolism (Cato et al., 1997; Fitzsimons & Collins, 1997).

Individuals who have contracted HIV and AIDS may eventually require invasive medical procedures (Wastell, Corless & Keeling, 1996). Midazolam may be used as a sedative for many of these procedures including laporoscopies, lumbar punctures, bronchoscopies, biopsies, and colonoscopies. In a recent clinical report, a patient prescribed the protease inhibitor saquinavir experienced profound sedative effects after the intravenous administration of five milligrams (mgm) of midazolam for a bronchoscopy. This individual subsequently required a midazolam reversal agent, Flumazenil 300 micrograms to recover (Merry et al., 1997). These authors suggested that because saquinavir and midazolam use the P450 3A isozyme for metabolism, there was an inhibition of the biotransformation and clearance of midazolam.

Rational and Significance

Anesthesia providers in the military and civilian sectors administer midazolam to a wide variety of patients. To provide safe and effective care it becomes essential to understand the type and duration of effect a medicine will provide. Investigation into the

interaction of midazolam and the protease inhibitor ritonavir is necessary to prevent the possibility of a negative patient outcome. Furthermore, these results may provide insight into the amount of time required between doses of ritonavir and midazolam. This knowledge may prove to be valuable information in a clinical or an outpatient setting to a patient currently receiving ritonavir.

Purpose of the Study

The purpose of this study was to identify through an in vitro experimental method how the metabolism of midazolam is affected in the presence of ritonavir. This study used cadaver liver microsomes and not human subjects. These results can be used to better understand the interactions involved in the metabolism of midazolam in the presence of protease inhibitor ritonavir.

Problem Statement

The wide spread use of midazolam in anesthesia and the increasing need for the protease inhibitor ritonavir to treat HIV and AIDS may lead to congruent administration of both these drugs. Clinical evidence demonstrates that negative outcomes have resulted from the administration of midazolam in the presence of a protease inhibitor (Merry et al., 1997). No studies to date have specifically addressed the interactions of these drugs in combination. Thus, a need to investigate the rate of metabolism of midazolam in the presence of the protease inhibitor ritonavir exists. The results of such a study can be used to better understand the effects of midazolam in the presence of the protease inhibitor ritonavir.

Null Hypothesis.

The null hypotheses for this study is:

There is no change in the rate of metabolism of midazolam in the presence of ritonavir when compared to midazolam alone as measured by the formation of midazolam metabolites.

Dependent Variable.

The dependent variable for this study is:

The rate of metabolism of midazolam as measured by the rate at which the first metabolite, α-hydroxymidazolam, is formed.

Independent Variables.

Independent variables for this study are ritonavir at the concentrations of 0.0 μM, 0.005 μM, 0.01μM, 0.05 μM and 0.1 μM.

Conceptual Framework

The conceptual framework for this study is based on the biotransformation of drugs in the body. Two phases are identified in this process, although a drug does not necessarily utilize both phases. During phase I reactions, a polar functional group is either added or revealed through oxidative, reductive, and hydrolytic reactions which inactivate the pharmacologic actions of the parent compound. Phase II reactions are identified as conjugation reactions, which result in a covalent bond between a functional group and an acetate, amino acid, glucuronic acid, glutathione, or sulfate group. This forms a highly polar compound, which is generally inactive and excreted in the urine.

Despite the fact that all tissues in the body are capable of some metabolism of xenobiotics, the liver is the main site of biotransformation (Katzung, 1998).

The system of biotransformation is known as either the mixed function oxidase system or the cytochrome P450 system. The system contains hemeproteins, which in their reduced (ferrous) form will bind to carbon monoxide and absorb light at 450 nM maximally, thus P450. This system of complex enzymes and pigmented hemeprotiens catalyzes most of the bodies oxidative and some of the reductive biotransformations. The P450 enzyme contains a hemeoprotein that combines with a drug to form an enzymatic complex. This complex is reduced by an electron, which is transferred from nicotinamide-adenine dinucleotide phosphate (NADPH) to a flavoprotein reductase. The next step is the transfer of a second electron via the flavoprotein reductase, which reduces molecular oxygen to form an activated oxygen-cytochrome and substrate complex. In the final process, one atom of oxygen is released as water and the second oxygen is transferred to the substrate (Katzung, 1998).

The P450 system is a super-family of related enzymes, all with unique functions that metabolize certain parent compounds. Specifically, P450 contains an enzyme subfamily known as cytochrome 3A (CYP 3A). These enzymes have been identified as responsible for the metabolism of midazolam and include CYP3A3, CYP3A4 and CYP3A5 (Wandel et al., 1994). Although many different enzymes have been identified in the mixed function oxidase system, a great deal of drugs administered use the CYP 3A isozyme found in the liver for metabolism.

The fact that many drugs use the same system for biotransformation sugests that an interaction between drugs is probable. Many drugs such as erythromycin and midazolam use the same enzymatic pathway (CYP3A) resulting in inhibition of their biotransformation (Hiller, Olkkola, Isohanni & Saarnivaara, 1990). Barry, Gibbons, Back and Mulcahy (1997) suggest that competition for the same substrate binding site is probably the most prevalent mechanism of inhibition. Eagling, Back and Barry (1997) demonstrated that the protease inhibitors ritonavir, saquinavir and indinavir inhibit the P450 isoforms. Koudriakova et al. (1998) revealed that CYP3A plays an essential role in the metabolism of ritonavir via human intestinal microsomes, with a K_m value <0.1 μM. Additionally, it was demonstrated that the metabolism of ritonavir in liver and enterocyte microsomes was associated with the inactivation of CYP3A. This also helps to explain the improved bioavailability and pharmacokinetics of ritonavir and the sustained elevated blood levels of other concomitantly administered drugs, which are substrates of the CYP3A.

Definitions

The following terms are used in this study.

α-hydroxymidazolam. The metabolite formed by the hydroxylation of α or the one carbon in midazolam. This is the prominent metabolite in the biotransformation of midazolam (Thummel et al., 1994a).

4-hydoxymidazolam. The metabolite formed as a result of hydroxylation of the 4th carbon of midazolam. (Thummel et al., 1994a).

Antegrade amnesia. Amnesia which takes place after the incident that involves the incident itself (Omoigui, 1995).

Antiviral. A substance that has properties that are aimed at preventing viral replication (Katzung, 1998).

Bioavailability. The fraction of unchanged drug reaching the systemic circulation following administration by any route (Katzung, 1998).

Biotransformation. The conversion of drugs from one form to another within an organism associated with a change in pharmacologic activity (Katzung, 1998).

Cmax. The maximum plasma concentration of a substance (Katzung, 1998).

Half-life (t 1/2). The time in hours required for the drug concentration in the body to decrease by 50% (Katzung, 1998).

High Performance Liquid Chromatography (HPLC). A process of separating chemical substances by differential movement through a stationary and mobile two-phase system. Material separated is injected through a column of a chosen absorbent and substances least absorbed appear first and the more absorbent emerge later (Willing & Tse, 1988).

In vitro. In an artificial environment; observable in a test tube (Miller & Keane, 1987).

In vivo. Within the living body (Miller & Keane, 1987).

K_i. The inhibition constant for Michaelis-Menten kinetics, which describes the ability of a drug to inhibit the catalysis of another drug. This value identifies the rate at which 50% of a xenobiotic's metabolism is inhibited. (Fabre et al., 1988)

K_m. The Michaelis constant describing an enzyme's affinity for a certain substrate. This value is equivalent to ½ maximal velocity in the catalysis of a chemical reaction (Katzung, 1998).

Microsomes. Small spherical vesicles obtained from the endoplasmic reticulum after disruption of cells and ultracentrifugation (Miller & Keane, 1987).

NADPH. Nicotinamide-adenine dinucleotide phosphate is a reduction agent. It is used by the P450 system in the metabolism of midazolam (Miller & Keane, 1987).

Pharmacodynamics. A drug's action on the body (Katzung, 1998).

Pharmacokinetics. The process of absorption, distribution, and elimination determine how rapidly and in what concentration and for how long the drug will appear at the target organ (Katzung, 1998).

Protease inhibitors. Drugs that inhibit the HIV protease enzyme that processes the viral proteins essential for the completion of the viral life cycle, thus decreasing the production of more infectious virons (Deeks et al., 1997).

Therapeutic index. The [toxic]/[therapeutic] ratio which estimates the margin of safety of a therapy (Katzung, 1998).

V max. The maximal velocity of biotransformation for a specific drug (Katzung, 1998).

Volume of Distribution (Vd). The amount of drug present in the body in relation to the concentration of the drug in the plasma expressed in liters (Katzung, 1998).

Xenobiotic. A pharmacologically active substance not endogenously produced and thus foreign to an organism (Katzung, 1998).

Assumptions

The assumptions of this study were:

1. The assay developed by Hinkle (1997) accurately identifies the rate of the metabolism of midazolam in the presence of ritonavir.

2. The CYP3A enzymes found in the cadaver livers are comparable to the general population. Studies have demonstrated that the range of microsomal CYP3A content of an individual may vary 10-100 fold (DeWaziers, Cunenc, Yang, Leroux, & Beaune, 1990; Guengerich & Turvy, 1991; Kronbach et al., 1989; Watkins et al., 1985).

3. An assumption is made that these results approximate those of an in vivo experimental model. The in vitro design of this study provides results to the microsomal samples available to this study, and not necessarily to an in vivo model.

4. Midazolam concentrations used were within the therapeutic range of the general population. This study utilized midazolam substrate at the concentrations 163-3909 ng/ml, within the expected intravenous range of 0.15mg/kg. These figures are based upon the therapeuitc range of 300-1400 ng/ml of midazolam for a healthy individual with a weight of 55-77 kg (Heizman, Eckert & Zeigler, 1983).

Limitations

The limitations of this study were:

1. The use of liver microsomes from only four human cadavers in a pooled fashion may limit the applicability of this study to the general population. Several studies have identified that microsomal CYP3A4 content may vary as much as 100 times in the

general population (DeWaziers et al., 1990; Guengerich & Turvy, 1991; Kronbach et al., 1989; Watkins et al., 1985).

2. The microsomes may have been exposed to drugs or environmental factors that effect CYP3A unbeknownst to us.

3. This study utilized ritonavir at the subtherapeutic concentrations of 0.005 μM, 0.01μM, 0.05 μM and 0.1 μM due to the fact that ritonavir demonstrated complete inhibition of α-hydroxymidazolam metabolite at higher concentrations.

CHAPTER II. REVIEW OF LITERATURE

Midazolam Profile

Midazolam is a short acting benzodiazepine with a wide range of effects. Chemically, midazolam is described as a 1,4 benzodiazepine derivative with an imidazole ring at the 1,2 position. Its quick onset as compared to other benzodiazepines can be attributed to the fact that it can change its lipid solubility within different pH environments (Smith, Eadie & Brophy, 1981). This drug is packaged at a pH of three, making it water-soluble and facilitating its handling out of the body. Once infused intravenously, it enters the body pH of 7.4 and becomes lipid soluble, thus facilitating its passage through the blood brain barrier (Gerecke, 1983). The pharmacodynamics of midazolam involves its actions on the gamma amino butyric acid (GABA) receptor. The GABA system is inhibitory to the central nervous system (CNS) and helps coordinate responses. The effects are that if the $GABA_A$ receptor is stimulated the rest of the CNS is inhibited.

Dundee and colleagues (1984) describe midazolam as an anxiolytic and sedative with anticonvulsant, sleep inducing, and muscle relaxing properties. It has a short duration of action and produces minimal cardiorespiratory changes. Some side effects of midazolam have included nausea after intramuscular (IM) or intravenous (IV) administration, and apnea at high doses. Therapeutic trials of midazolam demonstrated its use as an induction agent for general anesthesia and also as an intravenous sedative hypnotic for local anesthesia. It was also shown that midazolam can cause a definite

decrease in the tidal volume which is compensated for by increase in respiratory rate. Midazolam causes a central respiratory depression with a loss of respiratory drive with subsequent anoxia and death if not reversed.

Midazolam Metabolism

The cytochrome P450 family plays a role in the metabolism of many drugs including midazolam and protease inhibitors. Thummel et al. (1994a) found that midazolam clearance in the body is due to metabolic biotransformation catalyzed by the P450 CYP3A enzymes. This fact was further confirmed and elaborated upon by Fabre et al. (1988) who identified the P450 system as a major contributor to the metabolism of midazolam. In fact, a specific isozyme of this system that is involved in as much as 60 percent of metabolism was identified to be CYP3A4.

The metabolism of midazolam was described by Bauer et al. (1995) as a process that involves hydroxylation via the liver cytochrome P450 3A4 to form the metabolite α-hydroxymidazolam. This metabolite is further conjugated by glucuronides in the liver and is eliminated by kidney glomerular filtration and tubular secretion. Midazolam has been used as a probe for the P450 3A system, as shown in a study by Thummel et al. (1994b) who identified CYP3A activity in liver transplant patients.

A study by Wandel et al. (1994) found that cytochrome 3A3 and 3A5 are also involved in the metabolism of midazolam. Other factors identified in metabolism of midazolam include the dose of the drug that may inhibit the rate of its metabolism. It is reasonable to predict that if a specific drug in some way inhibits the P450 CYP3A family

of enzymes, there will be an increase plasma concentration of drugs that are metabolized and eliminated by this system. Bauer et al. (1995) found that accumulation of conjugated metabolites of midazolam yielded prolonged sedation requiring the administration of a benzodiazepine-receptor antagonist flumazenil.

Midazolam and Protease Inhibitors

With the establishment of the cytochrome P450 oxidase system as the major method of metabolism of midazolam, attention is now turned towards the protease inhibitors. Ritonavir, a protease inhibitor, assists patients with HIV antibody and AIDS by helping to prevent the further spread of the virus (Kempf et al., 1997). After the HIV virus invades a cell it goes through several processes. One of the latter steps of this process involves the breaking of long protein chains of the virus so that they can be packaged to complete the viral life cycle. The HIV protease enzyme cuts the long chain into shorter infectious products. If this is not accomplished, the long protein fragments are inactive and inhibits the spread of infectious viral progeny. Protease inhibitors such as ritonavir prevent the HIV protease from cutting these long HIV protein chains.

Protease inhibitors have wide ranging pharmacodynamic and pharmacokinetic properties that have limited their development (Deeks et al., 1997). They tend to be problematic from a tolerability perspective due to their side effects. Ritonavir side effects may include nausea, anorexia, diarrhea, asthenia, headaches, taste disturbances and circumoral parasthesia. The degree of severity of these side effects is related to plasma concentration of the drug, which is highest during initial dosing. For some patients, symptoms do subside with time.

Protease inhibitors are metabolized by the P450 oxidase system. In a study by Fitzsimmons and Collins (1997), the protease inhibitors saquinavir and indinavir were shown to use the P450 CYP3A4 system. They further demonstrated that the antifungal drug ketoconazole decreased the rate at which saquinavir metabolites were formed, thus slowing metabolism and elimination. Cato et al. (1997) revealed that the pharmacokinetics of ritonavir were altered by flucanazole, an antifungal drug. Kunze, Wienkers, Thummel and Trager (1996) showed flucanazole as an inhibitor of CYP3A. In the presence of flucanazole, there were increases in maximum plasma concentrations of ritonavir (Cato et al., 1997).

Protease inhibitors are sometimes given in combination to help bioavailability and enhance effects when treating HIV. For example, ritonavir is known to have potent inhibitory effects on the P450 3A4 system and as such helps to improve the bioavailability of the protease inhibitor saquinavir (Merry et al., 1997). Thus, saquinavir can overcome the large first-pass effect in the liver.

Ritonavir is specifically mentioned by Deeks et al. (1997) as both an inducer and inhibitor of the cytochrome P450 metabolic pathway. Plasma concentrations of many drugs to include midazolam are expected to be increased. As such, adverse and unexpected reactions can be anticipated. Eagling et al. (1997) found that protease inhibitors affect the cytochrome P450 system with a potential for significant drug interactions. This study further found that of all the protease inhibitors, ritonavir has the greatest effect on the CYP3A4 family of enzymes used for metabolism.

Koudriakova et al. (1998) revealed that CYP3A plays an essential role in the metabolism of ritonavir via human intestinal microsomes, with a K_m value <0.1 μM. Additionally, it was demonstrated that the metabolism of ritonavir in liver and enterocyte microsomes was associated with the inactivation of CYP3A. This also helps to explain the improved bioavailability and pharmacokinetics of ritonavir and the sustained elevated blood levels of other concomitantly administered drugs which are substrates of the P450 CYP3A system.

There is little information on the actual effects of midazolam use in combination with protease inhibitors. Although the U. S. Food and Drug Administration (FDA) has issued warnings discouraging there combined use, it is possible that these two drugs may be used inadvertently together (FDA, 1997). In fact, one such case was recently reported in which an individual on the HIV protease inhibitor saquinavir was administered midazolam and did not wake spontaneously. This individual required a reversal agent for midazolam, flumazenil, to recover and was not free of the sedative effects for five hours (Merry et al., 1997). Moreover, if drugs have substrates of a common P450 enzyme such as CYP3A and are administered in combination, it is possible that the elimination of at least one of those drugs would be prolonged.

CHAPTER III. METHODOLOGY

Overview

The general procedures of this experimental design have been described in many pharmacology studies. This specific assay was developed by Hinkel (1997), who provided information regarding gender related differences in the metabolism of midazolam. Slight modifications to this assay were made to make the design more applicable to this study. Human microsomes were prepared and pooled in an effort to provide a homogenous mixture of the P 450 isozymes found in the general population. This is accomplished in an effort to decrease the likelihood a unique enzymatic characteristic found in one individual would manifest itself and bias the results. High performance liquid chromotography (HPLC) was utilized to identify the velocity at which midazolam metabolite formation occurs. The protease inhibitor ritonavir was added to the midazolam to measure the change in the rate of metabolite formation.

Investigational Review Board

The Uniformed Services University of the Health Sciences Investigational Review Board (IRB) approval for this study was provided under exempt status. The protocol identification of this study is listed as T06157. Explicit identification information of the cadaver liver donors is kept strictly confidential and they remain completely anonymous.

Microsome Extraction

Microsomes for this study were extracted from cadaver livers obtained through Washington Regional Transplant Consortium, Washington, DC. These livers were not

suitable for transplantation but are useable for scientific research. Data describing each liver sample was reviewed for possible drug exposure that might affect the results of this study. The livers have a code number for tracking the descriptive data with the sample. No other identification of the source of the livers is possible. Microsomes were extracted from the human liver samples following the protocol for extraction. The four microsome extracts were then pooled after determining similar activity, followed by measuring the protein concentration using the Bio-Rad Protien Assay, BioRad Laboratories (Richmond, CA) with bovine serum albumin as a standard. The microsomes were stored at -80^0 Celsius until used.

Assay

The assay developed by Hinkle (1997) is selected due to its similarity to this study. Comparable sample preparations, incubation conditions, and microsome concentrations were used for this experiment.

Chemicals and Reagents

Materials utilized in this investigation are listed in Appendix A. Midazolam, and α-hydroxymidazolam, were a gift from F. Hoffman-LaRoche, A. G. (Basel, Switzerland). The internal standard lorazepam was provided by Sigma Chemical Co. (St. Louis, MO). HPLC grade acetonitrile and water was provided by Fisher Scientific (Pittsburgh, PA) and formic acid was provided by Aldrich Chemical Co. (Milwaukee, WI). Constituents of the NADPH generating system were purchased from Sigma Chemical Co. (St. Louis, MO).

Standard Solutions

Standard stock solutions of midazolam and its metabolite were prepared at a concentration of 0.32 mM in ethanol. Serial dilutions of the 0.32 mM standard were used to make the appropriate working solutions of midazolam. A standard stock solution of lorazepam was prepared at 3.11 mM in ethanol and further diluted to prepare the working solution at 5.0 μM (see Appendix B). Stock and working solutions were stored at 5° Celsius for the duration of the study, which lasted approximately two months.

Calibration Curves

The calibration curve was prepared by using a ratio of midazolam to α-hydyoxymidazolam of 10:1. The concentrations of midazolam were 0.5, 1.0, 3.0, 6.0 and 12.0 μM. Two quality control points were added with midazolam concentrations of 0.8 and 0.05 mM as a working solution. The pooled microsome suspension without the generating mixture was inoculated with each of the above mentioned concentrations and 200 μL of lorazepam (5 μM), then extracted per standard procedure. No incubation was done to these samples. There were seven standard curve points added to the beginning of each HPLC run (see Appendix C). Calibration curves were generated by least-square regression analysis of the analyte/internal standard peak-height ratio versus the concentration of the analyte.

Pilot Study

After all components and tools for the study were assembled and made ready, the experimental design was tested. The 4-hydroxymidazolam metabolite was not used in

this study due to its small signal peak, making it difficult to identify at levels of inhibition seen with ritonavir. The α-hydroxymidazolam was visualized due to its stronger signal. The pilot study consisted of the incubation of midazolam with the protease inhibitor ritonavir in various concentrations below and including the Cmax. Cmax steady state for a 600mg standard dose is listed as 11.2 μg/ml (Physicians' Desk Reference, 1999). Therapeutic doses of ritonavir demonstrated complete inhibition of midazolam metabolites. After further analysis, it was revealed that in order to properly identify the Ki of ritonavir in regards to midazolam, subtherapeutic concentrations were necessary. The concentrations of ritonavir utilized were:

0.005 μM ($3.6x10^{-3}$μg/ml)

0.01μM ($7.21\ x10^{-3}$μg/ml)

0.05 μM ($3.6\ x10^{-2}$μg/ml)

0.1 μM ($7.21\ x10^{-2}$μg/ml)

Experiment

The experimental design consisted of four broad phases.

1. Preparation of the microsomes
2. Timed incubation with the midazolam and protease inhibitor
3. Extraction of the metabolites from the remaining proteins
4. HPLC analysis.

The substrate concentrations were 0.5, 1.0, 3.0, 6.0 and 12.0 μM. Each of the five substrate concentrations were incubated with the protease inhibitor ritonavir

concentrations of 0.0, 0.005, 0.01, 0.05 and 0.1 μM. Each substrate concentration was run in quadruplicate, to yield 100 data points plus controls and standards.

Human Liver Microsome Information

Microsomes for this study were extracted from cadaver livers obtained through Washington Regional Transplant Consortium, Washington, DC. These livers were not suitable for transplantation but are useable for scientific research. Demographic data for these samples are listed in table 1.

Table 1.

Human Liver Microsome Data

Identification Number	Sex	Age	Medication
HLA 8	Male	73	Diltiazem for 15 years
HLA 10	Female	56	None
HLA 11	Female	16	Imipramine for 2.5 years
HLA 20	N/A	N/A	Not available

Preparation of Microsomes

Microsomes were prepared to a concentration of 10.73 mg/ml; analyzed with a BioRad protein assay. The microsomes were stored in liquid nitrogen and thawed prior to use. When the experiment proceeded, 18.6 μL were diluted by phosphate buffer to yield a final protein concentration of 0.2 mg protein/ml. Each microsomal suspension was warmed in a shaking water bath at 37^0 Celsius for three minutes after adding 10 μL of an NADPH generating system to catalyze the experiment. The NADPH generating

system was prepared by mixing 846 mg glucose-6-phosphate (G6P), 252 mg NADP+, 2460 μL buffer and 540 μL glucose-6-phosphate-dehydrogenase (G6PD), totaling 3,000 μL. This amount was enough to run 300 samples.

Incubation Phase

Ten μL of the five concentrations of MDZ were added at zero minutes and the reaction was incubated at 37° C for 5 minutes, then stopped by plunging the test tubes into ice. Two hundred μL of lorazepam (5 μM) was added to each sample and standards after the tubes were in ice.

Extraction Process

Five ml of acetonitrile was added to the incubation medium and the tubes were vortexed for ten minutes. The tubes were centrifuged at 2,000g and 5° C. for ten minutes. All incubation media then were transferred to clean tubes and labeled. The tubes were evaporated to dryness with a speed vacuum apparatus. Twenty μL of acetonitrile: water (1:1 v/v) is added and the tubes vortexed for three minutes. Two ml of acetonitrile were added to each tube and the tubes were again vortexed for three minutes and then centrifuged at 2,000g and 5^0 C. for ten minutes. The contents of the tubes were transferred to clean tubes and labeled. The tubes were again evaporated to dryness with a speed vacuum apparatus. Following this, 20 μL of acetonitrile:water (1:1 v/v) was added to each test tube and vortexed for two minutes followed by the addition of 20 μL of water. The test tubes were then vortexed for two minutes and transferred to microvials and loaded onto the HPLC system.

HPLC Analysis

Separation of midazolam, α-hydroxymidazlam, ritonavir and lorezepam was accomplished through the use of a 150x2mm prodigy 5 μ ODS (3), 100 Å column by Phenomenex (Torrence, CA) using a linear gradient. Initial conditions were 15.85% ACN, (0.05% HCOOH): water. Ultraviolet detection was set to 220nm. Each sample required one hour to analyze on the HPLC system. The peaks of the midazolam, α-hydroxymidazolam and lorazepam were visualized on the graph. If the peaks were not visualized at the known retention times, hand integration was performed to quantify the results. The data was then analyzed using GraphPad Prism Trial Vrsion 3.00 for Windows, GraphPad Software (San Diego, CA) Copyright © 1994-1999 by GraphPad Software, all rights reserved; Microsoft EXCEL version 7.0 copyright ©1997, (Redmond, WA) and Statistical Package for the Social Sciences (SPSS) version 8.02 (Orem, UT).

Enzyme Kinetics

To quantify results in this study, Michaelis-Menten kinetics were applied. The equation for Michaelis-Menten follows (Champe & Harvey, 1994).

$$v_o = \frac{V_{max}[S]}{K_m[S]}$$

Where: v_o = Initial velocity.

Vmax = Maximal velocity.

K_m = Michaelis constant.

[S] = Substrate concentration.

The Michaelis-Menten equation was utilized to assist in the description of enzyme catalyzed reactions at various substrate (midazolam) concentrations, the maximal biotransformation velocity for an enzyme (Vmax) and rate constants (K_m). In order to avoid the difficulty plotting curvilinear data of enzyme catalyzed reactions, the biochemists Lineweaver and Burk (1934) rearranged the Michaelis-Menten equation to a double reciprical plot, also referred to as a Lineweaver-Burk plot, as follows.

$$1/ v_o = K_m/(Vmax\ [S]) + 1/Vmax$$

This equation can be algebraically manipulated easily and is in the same format as the standard equation for a line ($y = mx + b$).

Data Analysis

Once calibration curves were established, the μM concentration of the experimental data set were calculated algebraically by substituting y in the standard equation of a line ($y = mx + b$), with the peak heights of the experimental data. Grubbs' test for statistical outliers was applied to the data to exclude extraneous points. The mean and standard deviation of the data was calculated and a 95% confidence interval

The inhibition constant (Ki) and percent inhibition for the α–hydroxylation of MDZ was calculated for each microsomal sample by nonlinear regression analysis using the software package Statisitcal Package for the Social Sciences (SPSS), (Orem, UT) and GraphPad Prism trial version 3.00 for Windows, GraphPad Software (San Diego, CA) Copyright © 1994-1999 by GraphPad Software, all rights reserved. ANOVA factorial was used to test for significance of inhibition at $p<0.05$ (see Appendices D and E for

results). Regression analysis measures the averages between points and generates a curve that follows the average path. Identification of the Ki involved the graphing of 1/velocity versus 1/midazolam concentration to determine the slope of each line. Plotting the slopes versus the concentration of ritonavir on a graph creates a line with the corresponding "x" intercept of this line yielding the Ki.

CHAPTER IV. RESULTS

Introduction

This in vitro study suggests that the rate of metabolism of midazolam is inhibited by the protease inhibitor ritonavir at subtherapeutic concentrations as evidenced by a percent inhibition as high as 95% ($p < 0.05$). The non-competitive inhibition of ritonavir revealed a Ki of 0.00956 μM ($p<0.05$). The rate of metabolism of MDZ is decreased in a linear fashion with an increase in Ritonavir concentration. These findings are consistent with other Ki's identified in a study by Eagling et al. (1997). Merry et al. (1997) have reported that midazolam's sedative effects were prolonged in the presence of the protease inhibitor saquinavir. This study suggests the possibility of in vivo inhibition of midazolam metabolism and thus prolonged sedative effects of midazolam.

Metabolism of Ritonavir and Midazolam

The kinetic parameters (Km and Vmax) for this study were obtained through the use of human liver microsomes (see figures 1, 2 and table 2). The metabolic profiles of ritonavir and midazolam were observed in the form of peak heights through the use of HPLC analysis (see table 3). This provided the data necessary to quantify midazolam's primary metabolite, α-hydroxymidazolam.

Data was manipulated through the process of integration in an effort to exclude baseline waver. The peak heights measured were identified according to the retention times of α-hydroxymidazolam at 18.45 minutes and the internal standard lorazepam at 28.15 minutes (see figure 3).

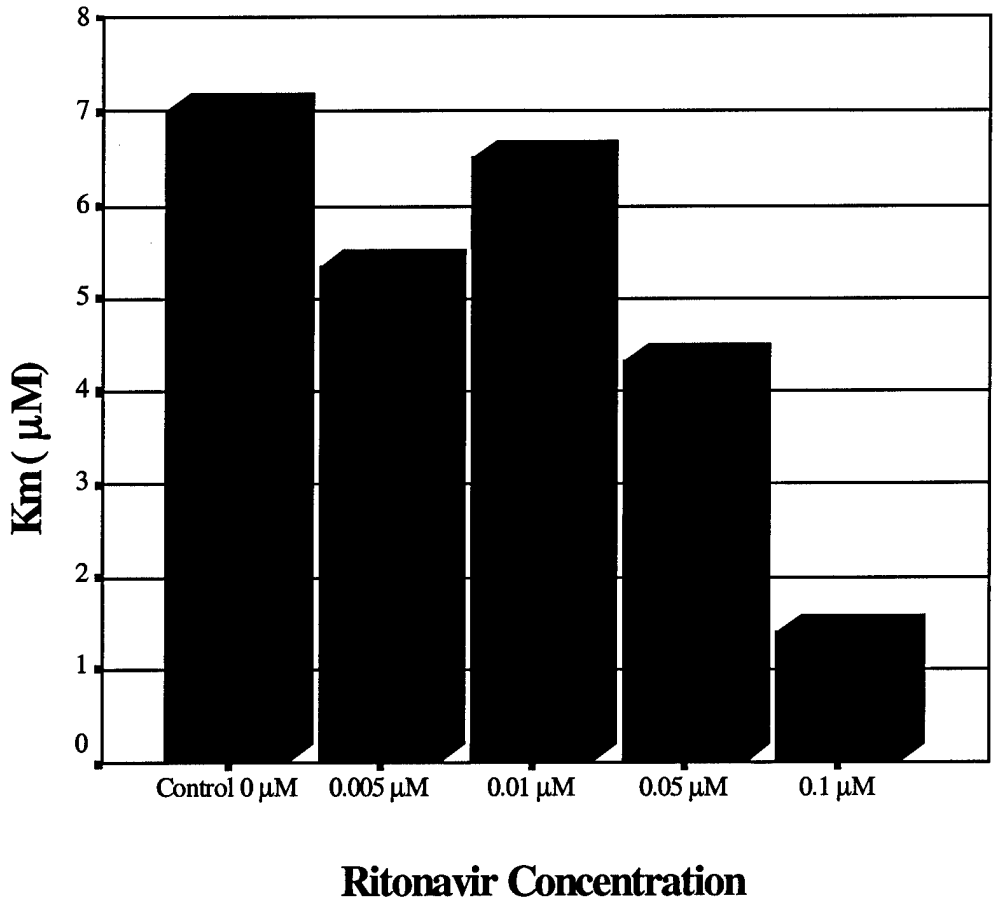

Figure 1.

Michaelis Substrate Affinity Constant (Km) Values.

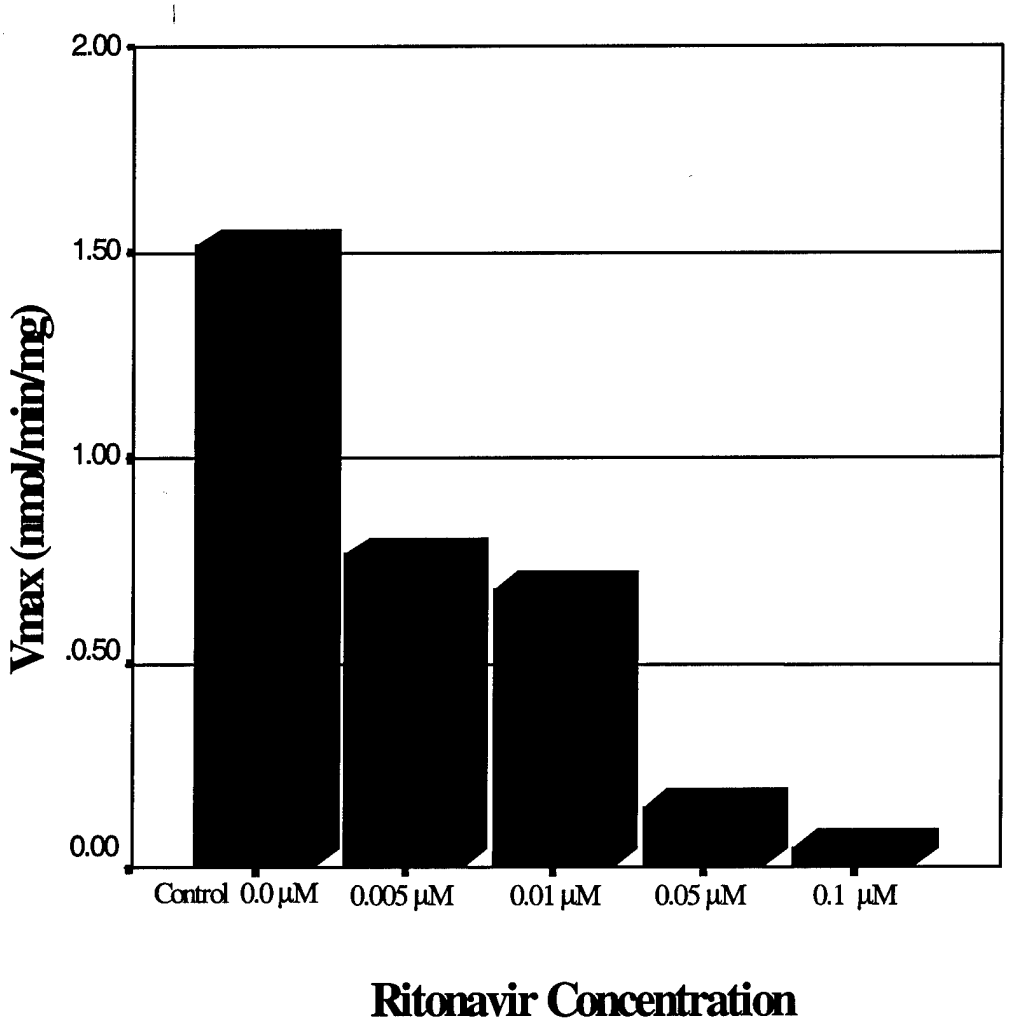

Figure 2.

Biotransformation Maximal Velocity (Vmax) Corresponding to Increasing Concentrations of Ritonavir.

Table 2.

Michaelis Substrate Affinity Constant (Km) and Biotransformation Maximal Velocity (Vmax) corresponding to Ritonavir concentration

	Ritonavir 0.0μM	Ritonavir 0.005μM	Ritonavir 0.01μM	Ritonavir 0.05μM	Ritonavir 0.10μM
Km (μM)	7.0152	5.3359	6.5034	4.3139	1.3899
Vmax (nmol/min/mg)	1.5152	0.7634	0.6803	0.1495	0.0496

Table 3.

Ritonavir (Peak Height/Internal Standard) Data in Relation to Increasing Concentrations of Midazolam

	0.5μM Midazolam	1.0μM Midazolam	3.0μM Midazolam	6.0μM Midazolam	12μM Midazolam
Ritonavir 0.000μM	97611.74	241902.1	511205.78	541275.71	830951.87
Ritonavir 0.005μM	67942.79	104512.88	299511.59	420224.89	637420.88
Ritonavir 0.010μM	50817.2	76398.11	233647.67	424651.55	458061.61
Ritonavir 0.050μM	16237.96	24565.5	48500.85	144199.63	142107.38
Ritonavir 0.100μM	14248.76	16881.82	27844.77	43849.93	76294.42

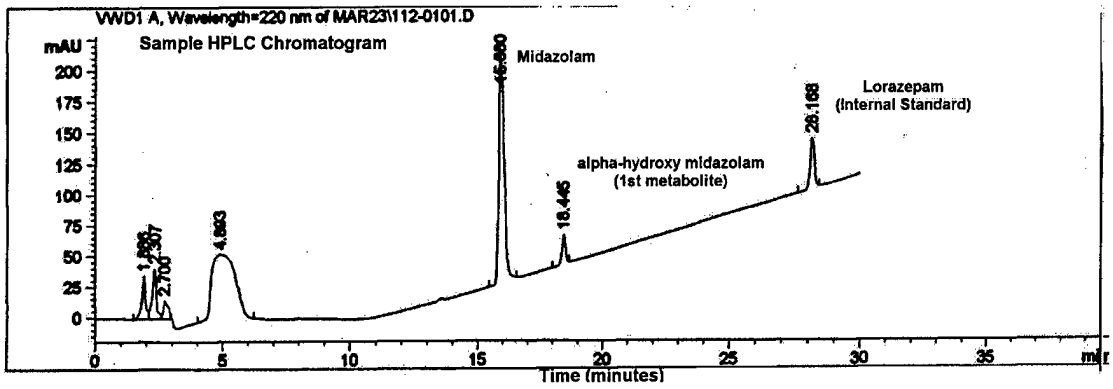

Figure 3.

Sample High Performance Liquid Chromatography (HPLC) Chromatogram.

In order to quantify α-hydroxymidazolam concentration via peak heights, the ratio of this metabolite was compared to internal standard curves. These were subsequently compared to standard peak heights of authentic standard curves prepared in a microsomal medium with a protein concentration of 0.2 mg/ml (see table 3). A standard curve can be derived through the use of the following formula.

α-hydroxymidazolam height / internal standard height = (Tg) x (C) + Yo

Where: Tg = Slope of standard curves

C = Concentration of calibrators

Yo = y intercept on y-axis

The concentration of α-hydroxymidazolam was then derived by solving for C as follows.

C = (α-hydroxymidazolam / internal standard – Yo) / (Tg)

Ritonavir Km, Vmax and Ki Results

This study utilized ritonavir at the subtherapeutic concentrations of 0.005 μM, 0.01μM, 0.05 μM and 0.1 μM due to the fact that ritonavir demonstrated complete inhibition of α-hydroxymidazolam metabolite at higher concentrations in pilot studies. Four replicates were accomplished for each concentration. The mean of the replicates were then used as the velocity of metabolite formation for Lineweaver-Burke plots. These plots were then graphed as 1/velocity of metabolite formation along the y-axis and 1/[substrate] along the x-axis, which provided a series of slopes for midazolam at the

concentrations of 0.5, 1.0, 3.0, 6.0 and 12.0 μM (see figure 4). These slopes were subsequently used as y-axis coordinates along with substrate concentrations for x-axis coordinates. The line, which corresponds best to these coordinates on graph, produces an x-axis intercept, from which the Ki is derived. The Ki identified for ritonavir in this study is 0.0956 μM (see figure 5).

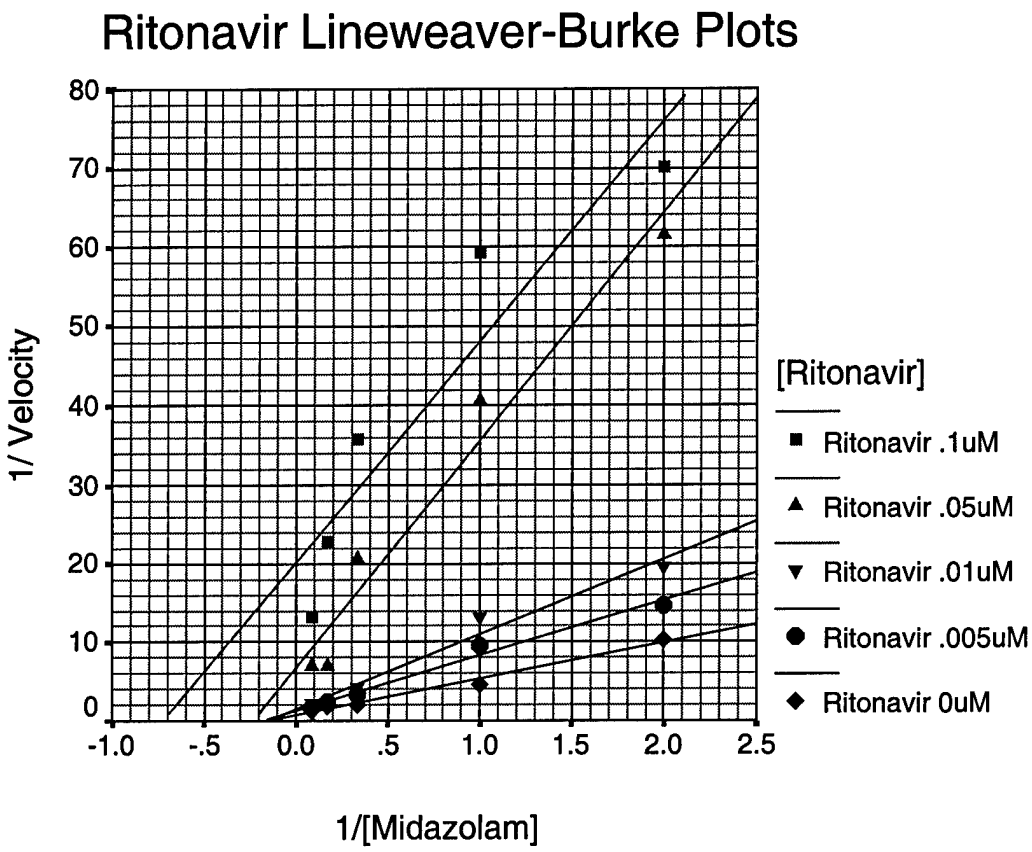

Figure 4.

Slopes Utilized to Derive Constant of Inhibition (Ki)

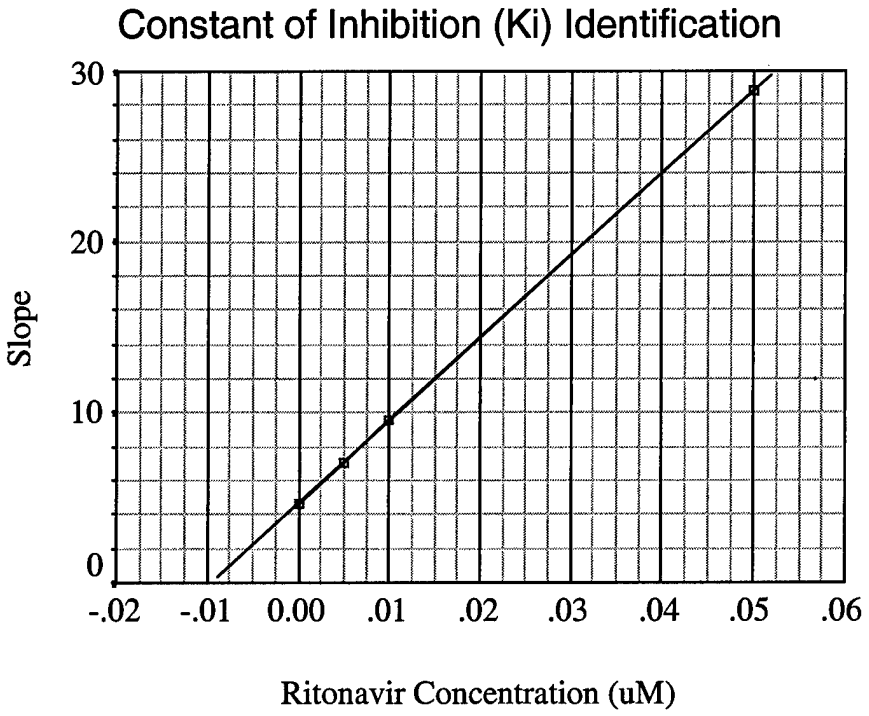

Figure 5.

Identification of Constant of Inhibition (Ki) at 0.00956 μM.

Percent Inhibition

Percent inhibition was calculated as a proportion of α-hydroxymidazolam formation at varying concentrations of midazolam (0.5, 1.0, 3.0, 6.0 and 12.0 μM) before and after the addition of protease inhibitor ritonavir at the varying concentrations of 0.005 μM, 0.01 μM, 0.05 μM and 0.1 μM. Significant inhibition at all concentrations was evident as high as 95% (see figures 6 and 7).

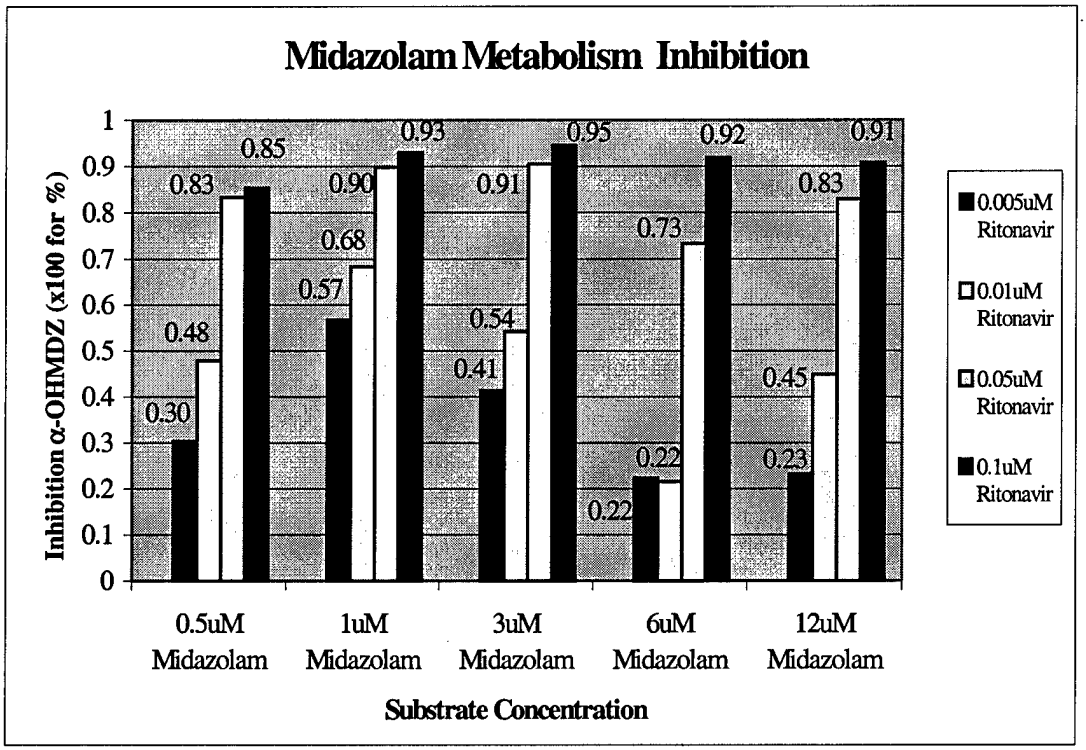

Figure 6.

Midazolam Metabolism Inhibition (x100 for percent)

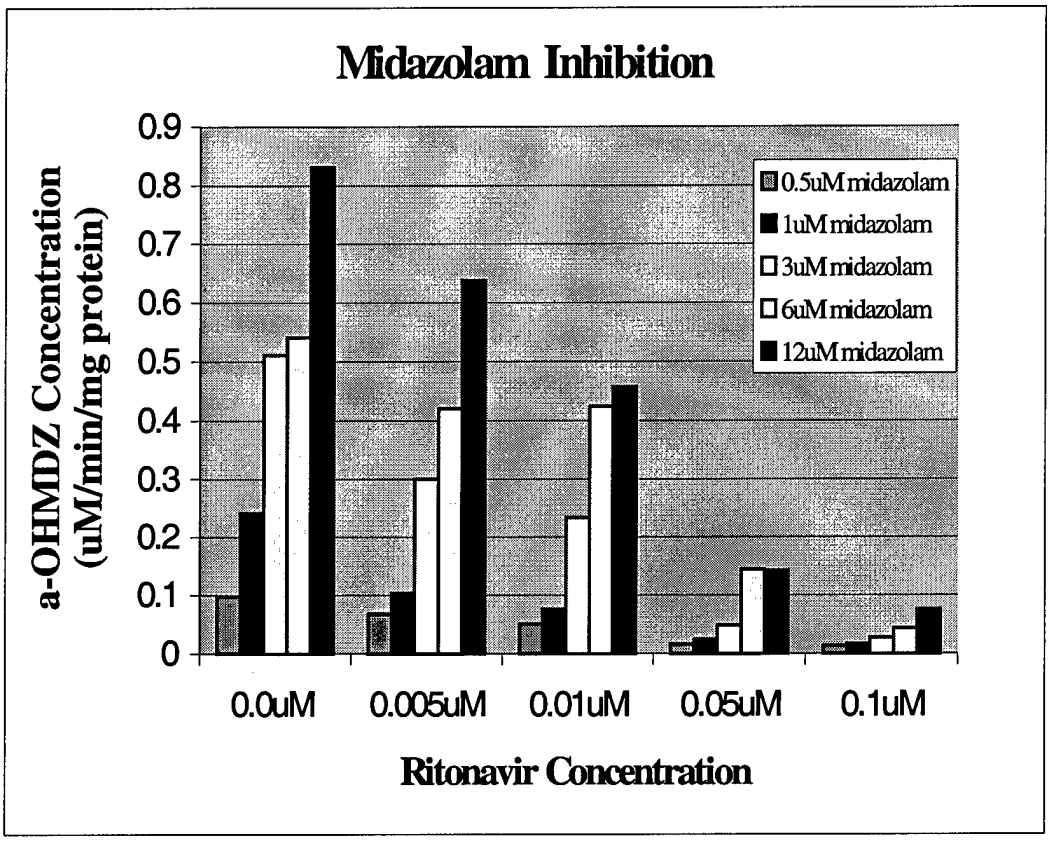

Figure 7.

Inhibition of Midazolam Metabolism Represented in Real Concentration.

CHAPTER V. DISCUSSION

Introduction

There are references made discouraging the coadministration of midazolam and ritonavir (FDA, 1997), yet no studies have specifically investigated the effect on the metabolism of midazolam. This in vitro study suggests that concerns regarding coadministration are valid and metabolism of midazolam is affected, as referenced by Eagling et al. (1997).

Results Comparison

Metabolism can be affected in a variety of ways. If a xenobiotic induces enzymes, there is generation of enzymes above the normal level thus increasing metabolic activities. Inhibition of enzymes limits the production of enzymes, thus decreasing metabolism of a xenobiotic. Inhibition is demonstrated in three ways. First, it could be mechanistic in which the enzyme is destroyed. Second, it could be competitive in which two substances compete for a site on an enzyme for metabolism. Third, it could be noncompetitive in which you have an allosteric control site (Katzung, 1998).

The Vmax values observed in this study increase in a concentration dependent fashion, while the Km value appears to remain constant when excluding the 0.10 μM data. This suggests a non-competitive type inhibition. The observed Ki of 0.00956 μM ($p<0.05$) demonstrates the extremely potent inhibition ritonavir has on the CYP3A system. Studies have demonstrated Ki values of ritonavir at 0.019 μM for testosterone

6β hydroxylation (Eagling et al., 1997), 0.05μM for methadone N-demethylation, and 0.02μM for buprenorphine N-dealkylation (Iribarne et al., 1997).

Ritonavir Effect on CYP3A

The result of this in vitro study adds to the body of knowledge that ritonavir is an extremely potent inhibitor of midazolam. Inactivation of both enteric and hepatic CYP3A with the initial transit of an oral dose acts to increase ritonavir bioavailability (Kudriakova et al., 1998). This suggestion was used to explain ritonavir's high bioavailability and increased inhibitory effects as compared to other protease inhibitors such as indinavir. The bioavailability of midazolam is thus likely to increase in vivo; subsequently it is likely that sedative effects may be enhanced or at least unpredictable. This may pose significant problems in the clinical setting. The widespread use of midazolam as a sedative hypnotic indicates a need to exercise greater care not administer these drugs in combination. This study suggests a possibility of a decrease in plasma clearance of midazolam, with subsequent prolonged hypnotic and respiratory depressant effects.

Data Discussion

In order to obtain the Ki of 0.00956, the 0.1 μM ritonavir data set was not utilized. After analysis, it was found that with this point included the Ki would be approximately 0.026 μM. Although still within acceptable range, the r^2 is 0.804. By excluding this data set in establishing the Ki, the r^2 is 0.999. When observing the slopes of all ritonavir datasets, it was evident that the 0.1 μM data set was not displaying the

same characteristics for determining inhibition type. Although close, it was not intersecting the x-axis in the same area as the other data points (see figure 4). A possible explanation for the anomaly at the 0.10 μM ritonavir data set includes system saturation. Further investigation is indicated; especially between the ritonavir concentrations of 0.05 and 0.1 μM to better evaluate the metabolic effects on midazolam. Another possible explanation for this variability may be laboratory error, such as pippeting, when preparing material for the 0.10 μM ritonavir data set.

Ritonavir results in this study differ also in the type of inhibition observed in other studies. Previous studies have demonstrated ritonavir to be a competitive inhibitor for the substrates buprenorphine and methadone (Iribarne et al., 1997). This effect is substrate specific. The non-competitive inhibition suggests that midazolam does not compete with ritonavir for active site binding, rather suggesting an allosteric control site.

Further investigation may be indicated between the ritonavir concentrations of 0.01 and 0.05 μM, as this study revealed a dramatic change in Vmax between these concentrations. This may provide a better understanding of midazolam inhibition in the presence of ritonavir and possibly identify the point at which enzyme saturation occurs.

As a suggestion of further research, conducting a study of two protease inhibitors in combination in the presence of midazolam may yield interesting results in regards to the rate of metabolism of midazolam. The relevance of such research is enhanced by the fact that protease inhibitors are being used in combination as therapy today against HIV.

Summary

Midazolam is a commonly used sedative hypnotic drug that may be inadvertently administered in combination with a protease inhibitor such as ritonavir. The data revealed in this in vitro study suggests that both ritonavir and midazolam utilize the CYP3A metabolic pathway for metabolism. Furthermore, ritonavir is an extremely potent inhibitor of that pathway, thus suggesting it may affect the metabolism of midazolam. This does present concerns to the clinician utilizing midazolam, in regards to delayed sedative hypnotic and respiratory depressant effects.

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APPENDICES

APPENDIX A

In Vitro Metabolism of Midazolam Materials:

In Vitro Metabolism of Midazolam Materials

1. Midazolam, Alpha-hydroxymidazolam.
2. Lorazepam.
3. HPLC grade acetonitrile.
4. Formic acid.
5. Nicotinamide-Adenine-Dinucleotide-Phosphate (NADPH) generating system: NADP+, Glucose-6-Phosphate (G6P), Glucose-6-Phosphate-Dehydrogenase (G6PD).
6. Human liver microsome samples that are pooled to a concentration of 10.73 mg/ml.
7. Protein assay: Bio-Rad Standard 11 dye, Bio-Rad, stock protein solutions ranging from 0.5-0.05 mg/ml, microliter plates.
8. 50 mM potassium phosphate buffer (pH 7.4).
9. Ethanol

APPENDIX B

In Vitro Metabolism of Midazolam Method

In Vitro Metabolism of Midazolam Method

1. Prepare stock solution of midazolam (MDZ) (3.2 mM):
    a. Formula weight MDZ = 325.8.
    b. Make 5 ml of stock solution.
    c. Weigh 5.213 mg MDZ in a volumetric flask.
    d. Add up to 5 ml ethanol.
    e. Store in refrigerator.
2. Prepare stock solution of α -hydroxymidazolam (3.2 mM):
    a. Formula weight = 341.8.
    b. Make 50 ml of stock solution.
    c. Weigh 5.469 mg α - hydroxymidazolam in a volumetric flask.
    d. Add up to 50 ml ethanol.
    e. Store in refrigerator.
3. Prepare 3.11 mM stock solution of lorazepam (internal standard).
4. Make MDZ dilutions from stock (10ml each) to use in incubations:
    a. 800 μM------- 2500 μL stock, 7500 μL distilled water.
    b. 600 μM-------- 1900 μL stock, 8100μL distilled water.
    c. 400 μM -------- 1250 μL stock, 8750 μL distilled water.
    d. 200 μM -------- 625 μL stock, 9375 μL distilled water.
    e. 100 μM -------- 313μL stock, 9687 μL distilled water.
    f. 50 μM --------- 156 μL stock, 9844 μL distilled water.

5. Make a mix of MDZ and it's metabolites to use to construct calibration curves:
    a. 1000 µL MDZ stock (3.2 mM).
    b. 1000 µL 1-Hydroxymidazolarn (3.2 mM).
    c. 1000 µL 4-Hydroxymidazolam (3.2 mM).
    d. 1000 µL distilled water.
    e. The resulting solution is 800 µM.
6. Take 4ml of the MDZ mix (800 µM) and put 1 ml and 1 ml in two separate tubes.
    a. Add 1ml water to one tube and add 3 ml water to the other tube.
    b. The result is 400 µM and 200 µM concentrations of the MDZ mix.
7. It is now required to continue making serial dilutions of the mix according to the following (4ml – 800 µM solution):
    a. 1 ml + 1 ml H_20 = 400 µM (2ml).
    b. 1ml + 3ml H_20 = 200 µM (4ml), take 2 ml from this to make next dilution.
    c. 1ml + 1ml H_20 = 100 µM (2ml).
    d. 1ml + 3-rnl H_20 = 50 µM (4ml), take 2 ml for next dilution.
    e. 1ml + 1 ml H_20 = 25 µM (2ml).
    f. 1ml + 4ml H_20 = 10 µM (5 ml), take 2 ml for next dilution.
    g. 1ml + 1ml H_20 = 5 µM (2 ml).
    h. 1ml + 4ml H_20 = 1 µM (5ml), take 2 ml for next dilution.
    i. 1ml + 1ml H_20 = 0.5µM (2ml).

j. 1ml + 4ml H_20 = 0. 1 μM (5ml), take 2 ml for next dilution.

k. 1ml + 1ml H_20 = 0.05μM (2ml).

l. 1ml + 4ml H_20 = 0.01 μM (5ml), take 2 ml for next dilution.

m. 1ml + 1ml H_20 = 0.005 μM (2ml).

n. 1ml + 4ml H_20 = 0.001 μM (5ml).

8. The pooled microsome samples are subjected to 5 different concentrations of MDZ (0.5, 1, 3, 6, 12 μM) and done in quadruplicate.
9. Perform protein assay on all microsome samples.
10. Prepare NADPH generating system:
    a. Mix: 650 μg G6P, 252 mg NADP+, 2490 μL buffer, 540 μL G6PDH, (556 μg/ml).
    b. This will total 30 μL, enough for 300 samples.
11. Add sufficient microsome suspension to each test tube to obtain a final protein concentration of 0.2 mμ/ml after dilution.
12. Base this on the calculated protein content of each microsome sample.
13. Add 10 μL of NADPH generating system and up to 0.980 μl of 50 mM potassium phosphate buffer.
14. Place test tubes in a shaking water bath for 3 min. at 37 C. Tubes used for standards should be kept on ice.
15. Add 10 μL of MDZ solution at 0 min. (The tubes used for standards will have added, then kept on ice without incubation).

16. Incubate tubes 5 min.
17. Plunge tubes into ice at 5 min.
18. Add 200 µL of the internal standard stock solution to each test tube (samples and standards).
19. Add 5 ml acetonitrile to each tube of incubation.
20. Vortex each tube for 10 min.
21. Centrifuge tubes at 2000 g; 5° Celsius for10 minutes.
22. Transfer to clean tubes and label.
23. Evaporate tubes to dryness with speed vac.
24. Add 20 µL acetonitrile:water (1: 1 v/v).
25. Vortex each tube for 3 min.
26. Add 2 ml of acetonitrile to each tube.
27. Centrifuge tubes at 2000 g, 5° Celsius, 10 min.
28. Transfer to clean tubes and label.
29. Evaporate tubes to dryness with speed vacuum.
30. Add 20 µL acetonitrile:water (1: 1 v/v) to each tube.
31. Vortex for 2 minutes at speed of 5.
32. Add 20µL water to each tube.
33. Vortex 2 minutes at speed of 5.
34. Transfer each sample from test tube to microvial.
35. Label and load onto HPLC system.

APPENDIX C

In Vitro Metabolism of Midazolam Chromatography

In Vitro Metabolism of Midazolam Chromatography

1. Involves the use of a Hewlett Packard 1050 HPLC system.
2. System fitted with micro bore tubing and Prodigy 5 μ ODS (3) 100 A column.
3. Flow rate = 0.2 ml/min.
4. Run time = 60 minutes.
5. Temperature = 35^0 C.
6. Injection vol.=10 μ L.
7. Lamp wavelength = 220 nm.
8. Solvent A = H_20.
9. Solvent B = 0.05% Formic acid in acetonitrile (pH 4.1).
10. Flow rate gradient:
    a. Time in minutes: Flow rate - 0:0.2, 2:0.2, 3.5:0.25, 5:0.25, 30:0.25, 32:0.25, 33:0.4, 39.8:0.4, 40:0.25, 45:0.25, 55:0.2.
11. Gradient conditions:
    a. Time in min: Percent formic acid in acetonitrile - 0: 15, 2:15, 3.5:15, 5:15, 30:45, 32:98, 33:98, 39.8:98, 40:98, 45:15, 55:5.

APPENDIX D

Statistical Data Results

Statistical Data Results

	Rit 0.000 μM	Rit 0.005 μM	Rit 0.010 μM	Rit 0.050 μM	Rit 0.100 μM
Variables					
Slope	4.6310 ± 0.31547	6.9894 ± 0.52600	9.5602 ± 0.86023	28.859 ± 3.1804	28.024 ± 6.1428
Y-intercept	0.66216 ± 0.32003	1.3057 ± 0.53362	1.4657 ± 0.87268	6.6940 ± 3.2265	20.165 ± 6.2317
X-intercept	-0.14298	-0.18681	-0.15331	-0.23196	-0.71956
1/slope	0.21594	0.14307	0.10460	0.034651	0.035684
95% Confidence Intervals					
Slope	3.6272 to 5.6348	5.3157 to 8.6632	6.8230 to 12.297	18.739 to 38.979	8.4775 to 47.570
Y-intercept	-0.35619 to 1.6805	-0.39230 to 3.0037	-1.3112 to 4.2425	-3.5726 to 16.961	0.33561 to 39.994
Goodness of Fit					
r^2	0.98627	0.98329	0.97629	0.96484	0.87402
Sy.x	0.50649	0.84452	1.3811	5.1063	9.8625
Is slope significantly non-zero?					
F	215.50	176.56	123.51	82.336	20.813
DFn, DFd	1.0000, 3.0000	1.0000, 3.0000	1.0000, 3.0000	1.0000, 3.0000	1.0000, 3.0000
P value	0.0007	0.0009	0.0016	0.0028	0.0197
Deviation fm zero?	Significant	Significant	Significant	Significant	Significant
Data					
Number of X values	5	5	5	5	5
Max # of Y replicates	1	1	1	1	1
Total # of values	5	5	5	5	5
# of missing values	0	0	0	0	0

APPENDIX E

Midazolam Statistical Information for Ki Identification

Midazolam Statistical Information for Ki Identification

Variables	
Slope	484.7 ± 1.874
Y-intercept	4.634 ± 0.04801
X-intercept	**-0.009560**
1/slope	0.002063
95% Confidence Intervals	
Slope	Perfect line
Y-intercept	Perfect line
Goodness of Fit	
r^2	**1.000**
Sy.x	0.07422
Is slope significantly non-zero?	
DFn, DFd	1.000, 2.000
Deviation from zero?	**Perfect line**
Data	
Number of X values	4
Maximum number of Y replicates	1
Total number of values	4
Number of missing values	0